Original Article

Detection of apoptosis by RT-PCR array in mefloquine-induced cochlear damage

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Abstract Objective To investigate the occurrence and possible mechanisms of apoptosis in cochlear epithelium and spiral ganglion neurons after mefloquine treatment. **Methods** We used quantitative RT-PCR apoptosis-focused gene arrays (96-well, 84 apoptosis related genes) to assess changes of gene expression in the cochlear basilar membrane (hair cells-supporting cells) and spiral ganglion neurons of rat cochlear organotypic cultures treated with 100 μM mefloquine for 3 h. **Results** Significant up-or down-regulation in gene expression was detected in 23 genes in the cochlear basilar membrane, and in 32 genes in the spiral ganglion neurons compared with time-matched controls. The responding genes could be classified as pro-or anti-apoptotic, and were mainly implicated in the Bcl-2, Caspase, Card, IAP, TNF ligand / TNF receptor, Death domain / Death effector domain, DNA damage / p53, and NF-kappa B families. Synthetic analysis suggested that these families could be revised to two major pathways mainly involved in the death receptor-mediated signaling pathway and apoptotic mitochondrial pathway. In addition, it was found that numerous anti-apoptotic genes such as Bcl2a1, Birc1b, Birc3, Birc4, Bnip1, Cflar, Il10, Lhx4, Mcl1, Nfkb1, Prlr, Prok2, and TNF were greatly up-regulated in the cochlear tissue, which might imply the co-existence of protective response in the cells at the early stage of mefloquine-induced damage.

Key words mefloquine; ototoxicity; apoptosis; cochlea; spiral ganglion neurons; hair cells; gene expression

Intrduction

Mefloquine is a potent antimalarial drug, widely used for prophylactic and chemotherapeutic treatment of malaria. As a synthetic analogue of quinine, the molecular formula, the range of clinical application, and the side-effect of mefloquine are similar to quinine. However, the neurotoxic and psychiatric side-effects of mefloquine are more severe than quinine which include depression, anxiety, paranoia, aggression, nightmares, insomnia, seizures, peripheral motor-sensory neuropathy, and many problems in the central nervous system.[1-10] Over the past years, there has been a growing concern among government agencies about the adverse ototoxic potential of mefloquine.[7, 9, 11-13] Based on these preliminary studies as well as limited clinical reports in the literature concerning the ototoxic and vestibulotoxic potentials, the specific aim of this study is to evaluate

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mechanisms of mefloquine caused apoptosis in the cochlea.

Methods

Animals

Fischer-344 (F344) rats at the age of postnatal day 3 were used. Experiments were performed according to the rules and regulations of the Institutional Animal Care and Use Committee of the State University of New York at Buffalo and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Cochlear membranous labyrinth dissection

Using techniques reported in our previous studies, postnatal day 3 F344 rats were decapitated and the cochlear basilar membrane and spiral ganglion neurons in Rosenthal's canal were microdissected out in Hanks Balanced Salt Solution (HBSS, Invitrogen).[14, 15] Briefly, the stria vascularis and spiral ligament were removed, and the whole cochlear basilar membrane along with the spiral ganglion neurons were dissected out under a dissection microscope in HBSS. To separate the cochlear basilar membrane containing the organ of Corti and the Rosenthal's canal containing spiral ganglion neurons, the whole Rosenthal's canal was further separated along the inner edge of the basilar membrane.

Mefloquine treatment

Cochlear basilar membrane and Rosenthal's canal were incubated at 37℃ and 5% CO_2 in a serum-free medium, with or without 100 μM of mefloquine, in 35 mm polystyrene dishes (Falcon, Becton Dickinson, Franklin Lakes, NJ) for 3 h. The serum-free medium consisted of bovine serum albumin [Sigma A-4919] 2 g, Serum-Free Supplement [Sigma I-1884] 2 ml, 20% Glucose [Sigma G-2020] 4.8 ml, Penicillin G [Sigma P-3414] 0.4 ml, 200 mM Glutamine [Sigma G-6392] 2 ml, and 1× BME [Sigma B-1522] 190.8 ml. Fifteen cochlear basilar membrane specimens containing the organ of Corti, or fifteen Rosenthal's canal specimens containing spiral ganglion neurons were treated in one dish. Mefloquine treated and non-treated specimens were pooled from 15 cochleae to generate one sample. Each sample was run separately for the qRT-PCR analysis. Five samples were tested, totaling 150 cochleae from 75 rats.

Quantitative RT-PCR apoptosis-focused gene arrays

In this study, we assayed the epithelium of basilar membrane containing the cochlear hair cells and supporting cells with or without mefloquine treatment using qRT-PCR array designed in 96-wells for the detection of 84 key apoptosis related genes. Sample preparation and the RT-PCR array determination were similar as described in our previous publications.[16-18] In brief, after 3 hours of culture with or without mefloquine, cochlear tissues were disrupted and homogenized in Buffer RLT. Total RNA was isolated and extracted using an RNA extraction kit (RNeasy Mini Kit, Qiagen) according to manufacturer's protocols. The solution containing extracted RNA was treated with RNase-Free DNase (Qiagen, Catalog# 79254) to remove DNA contamination. After the initial extraction, the RNA solution was cleaned up using RT2 qPCR-Grade RNA Isolation Kit (SuperArray, catalog # PA-001). The quantity of total RNA was evaluated with a spectrophotometer (Beckman Coulter DU 640). The amount of total RNA extracted from 15 cochleae was 507.6 ± 51.2 μg in epithelium of cochlear basilar membrane, and 291.2 ± 88.9 μg in spiral ganglion neurons respectively. After the RNA extraction and quality assessment, cDNA synthesis was performed using the kit of cDNA Synthasis Master Mix according to the manufacturer's protocols, and then cRNA amplification was performed using the kit of Amplification Master Mix in accordance with the manufacturer's protocols. The RT2 Profiler PCR Array (SABiosciences, Frederick, MD, USA) was used to measure the expression levels of apoptosis-related genes. Samples were prepared with the Master Mix and Template Cocktail and loaded into the 96-well qRT2-PCR array. RT2-qPCR arrays were run on BioRad MyiQ thermal cycler using SYBR Green detection. Cycle thresholds were determined for each gene using the instrument's software. Cycle threshold values were transferred into a Data Analysis Template Excel file (SuperArray) and ΔΔCt values determined for each gene in the mefloquine-treated and control array. Fold change in expression of each gene in Mefloquine was compared to Control array.

Results

Changes in expression of apoptosis related genes in

epithelium of basilar membrane

After 3 h of mefloquine treatment, expression changes were detected in 23 of the 84 studied genes. Expression was increased in 10 genes, but decreased in the other 13 genes. The rest 61 genes showed no significant change in comparison with untreated cochlear tissues. The 10 genes showing increased expression were *Bcl2a1, Birc3, Gadd45a, Il10, Mcl1, Nfkb1, Prok2, Pycard, Tnf, and Tnfsf6*, and those showing decreased expression were *Bid3, Birc4, Bnip1, Casp1, Casp7, Casp9, Cradd, Faim, Prlr, Sphk2, Tnfsf10, Tnfsf12, and Tradd*. These genes are relevant to multiple apoptotic signaling pathways, including the *Bcl-2* (*Bcl2a1, Bid3, Bnip1, Mcl1*), *Caspase* (*Casp1, Casp7, Casp9*), TNF ligand / receptor (*Faim, Tnf, Tnfsf6, Tnfsf10, Tnfsf12, Il10, Prok2, Sphk2, Tradd*), IAP (*Birc3, Birc4*), Death domain / Death effector domain (*Cradd, Tradd*), p53, DNA damage-induced apoptosis (*Casp7, Casp9, Cradd, Gadd45a, Mcl1, Nfkb1, Pycard, Tnf, Tnfsf6, Tradd*), NF-kappa B (*Bcl2a1, Il10, Nfkb1, TNF, Tnfsf6,*

Table 1 The functional grouping and fold changes of responding genes in cochlear epithelium by mefloquine injury were as described in table 1. The name of genes with bold italics written are anti-apoptotic genes, while the gene name with standard type are pro-apoptotic genes.

Gene	Description	Functional Gene Grouping	Fold changes
Bcl2a1	B-cell leukemia/lymphoma 2 related protein A1	Bcl-2 family	3.63
Bid3	BH3 interacting (with BCL2 family) domain, apoptosis agonist	Bcl-2 family	-3.14
Birc3	Inhibitor of apoptosis protein 1	IAP family	21.86
Birc4	Baculoviral IAP repeat-containing 4	IAP family	-2.14
Bnip1	BCL2/adenovirus E1B 19kDa-interacting protein 1	Bcl-2 family	-2.09
Casp1	Caspase 1	Caspase family / Card family	-2.43
Caps7	Caspase 7	Caspase family / p53 family	-2.28
Casp9	Caspase 9	Caspase family / p53 family / Card family	-2.08
Cradd	CASP2 and RIPK1 domain containing adaptor with death domain (predicted)	Death domain family / Card family / p53 family	-2.96
Faim	Fas apoptotic inhibitory molecule	TNF ligand / receptor family	-2.37
Gadd45a	Growth arrest and DNA-damage-inducible 45 alpha	p53 family	2.69
Il10	Interleukin 10	NF-kappa B family	5.68
Mcl1	Myeloid cell leukemia sequence 1	Bcl-2 family / p53 family	4.37
Nfkb1	Nuclear factor of kappa light chain gene enhancer in B-cells 1, p105	p53 family / NF-kappa B family	2.15
Prlr	Prolactin receptor	Cytokine receptor family	-2.34
Prok2	Homolog of mouse Bv8(Bombina variegata 8 kDa); prokineticin 2 precursor	TNF ligand / receptor family	6.6
Pycard	Apoptosis-associated speck-like protein containing a CARD	Death domain family / p53 family	2.18
Sphk2	Sphingosine kinase 2	TNF ligand / receptor family	-2.01
Tnf	Tumor necrosis factor superfamily, member 2	TNF ligand family / NF-kappa B family	19.08
Tnfsf10	Tumor necrosis factor (ligand) superfamily, member 10	TNF ligand family / NF-kappa B family	-3.55
Tnfsf12	Tumor necrosis factor ligand superfamily member 12	TNF ligand family / NF-kappa B family	-2.83
Tnfsf6	Tumor necrosis factor (ligand) superfamily, member 6	TNF ligand family / p53 family / NF-kappa B family	4.6
Tradd	TNFRSF1A-associated via death domain	TNF ligand /p53 / NF-kappa B / Death domain	-3.32

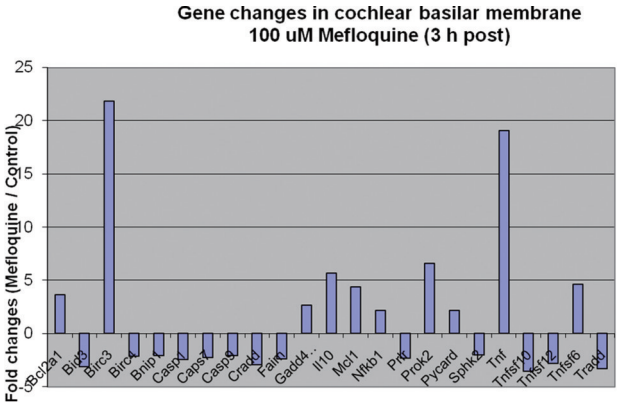

Figure 1 Significant changes in gene expression in cochlear epithelium 3 hours after 100 μm mefloquine treatment. The expression in 10 genes was increased, but the expression in another 13 genes was decreased

Tnfsf10, Tradd), Card (*Birc3, Birc4, Casp1, Casp9, Cradd, Pycard*), and Cytokine receptor families (Prlr). They are either pro-apoptotic (*Bid3, Casp1, Casp7, Casp9, Cradd, Gadd45a, Pycard, Tnfsf6, Tnfsf10, Tnfsf12, Tradd*) *or anti-apoptotic genes* (*Bcl2a1, Birc3, Birc4, Bnip1, Faim, Il10, Mcl1, Nfkb1, Prlr, Prok2, Sphk2, Tnf*) (Table 1)(Figure 1).

Changes in expressive of apoptosis related genes in spiral ganglion neurons.

In the tissue of spiral ganglion neurons, 32 genes showed changes in gene expression after mefloquine treatment, with 25 showing increased expression and 7 showing decreased expression. There was no expression change in the rest 52 genes. The 25 genes showing increased expression were Apaf1, Bcl10, Bcl2a1, Bcl2l11, Birc1b, Birc3, Casp14-predicted, Cflar, Gadd45a, Il10, Lhx4-predicated, Lta, Nfkb1, Prok2, Pycard, Ripk2, Tnf, Tnfrsf10b-predicted, Tnfrsf11b, Tnfrsf1a, Tnfrsf6, Tnfsf5, Tnfsf6, Trp63, and Trp73-predicted, and the 7 genes with decreased expression were Birc5, Card6-predicted, Casp9, Cradd, Tnfsf10, Tnfsf12, Tradd. Nineteen of these genes are pro-apoptotic (*Apaf1, Bcl10, Bcl2l11, Card6-predicted, Casp14-predicted, Casp9, Cradd, Gadd45a, Lta, Pycard, Tnfrsf10b-predicted, Tnfrsf11b, Tnfrsf1a, Tnfsf10, Tnfsf12, Tnfsf6, Tradd, Trp63, Trp73-predicted*) and the remaining 13 genes (*Bcl2a1, Birc1b, Birc3, Birc5, Cflar, Il10, Lhx4-predicated, Nfkb1, Prok2, Ripk2, Tnf, Tnfrsf6, Tnfsf5*) are anti-apoptotic genes. The multiple apoptotic signals are involved in the following pathways: the Bcl-2 family(*Bcl2a1, Bcl2l11*), Caspase family (*Casp14-predicted, Casp9*), IAP family (*Birc1b, Birc3, Birc5*), TNF ligand / receptor family (*Lta, Prok2, Tnf, Tnfsf5, Tnfsf6, Tnfsf10, Tnfsf12, Tnfrsf10b-predicted, Tnfrsf11b, Tnfrsf1a, Tnfrsf6, Tradd*), Death domain / Death effector domain family (*Cflar, Cradd, Tnfrsf11b, Tnfrsf6, Tradd*), Card family (*Apaf1, Bcl10, Birc3, Card6-predicted, Casp9, Cradd, Pycard, Ripk2*), p53 family and DNA damage-induced apoptosis (*Apaf1, Casp9, Cradd, Gadd45a, Nfkb1, Pycard, Tnf, Tnfrsf10b-predicted, Tnfrsf6, Tnfsf6, Tradd, Trp63, Trp73*), NF-kappa B family (*Bcl10, Bcl2a1, Cflar, Il10, Lta, Nfkb1, Ripk2, Tnf, Tnfrsf1a, Tnfrsf10b-predicted, Tnfsf6, Tnfsf10, Tradd*), and LIM-homeobox gene family(*Lhx4-predicated*) (Table 2)(Figure 2).

Discussion

RT-PCR (reverse transcription-polymerase chain reaction) arrays are the most reliable tools for analyzing the expression of a focused panel of genes for mRNA detection and quantification. Quantitative RT-PCR apoptosis-focused gene array is designed for analysis of expression of a panel of genes associated with numerous apoptotic biological pathways. In current apoptosis focused qRT-PCR primer set, the qPCR primer assay has been tested and validated for gene-by-gene expression analysis and microarray data validation. Except the housekeeping genes, the apoptosis RT-Profiler PCR array profiles the expression of 84 key genes involved in programmed cell death which covers the possessory apoptotic pathways in the current study. Of the 84 tested apoptosis-related key genes included in the RT2 Profiler PCR Array, 55 are pro-apoptotic genes and 29 are anti-apoptotic genes. Whether apoptotic or anti-apoptotic, changes in expression of these genes are generally indicative of cellular responses to stimuli of specific apoptotic signals. Therefore, the early change genes may indicate a response to the earliest apoptotic signals, and their apoptotic pathways may be involved in the primary apoptotic mechanisms. In contrast, the unresponsive genes can be taken into account as indecisive genes at the early stage, and their apoptotic pathways are supposed to be secondary apoptotic mechanisms in mefloquine-induced cell apoptosis in the co-

Table 2 The functional grouping and fold changes of responding genes in cochlear spiral ganglion neurons by mefloquine injury were as described in table 2. The name of genes with bold italics written are anti-apoptotic genes, while the gene name with standard type are pro-apoptotic genes

Gene	Description	Functional Gene Grouping	Fold changes	pvalue
Apaf1	Apoptotic peptidase activating factor1	Card family	2.52	0.0021
Bcl10	B-cell/lymphoma 10	Card family	2.18	0.0026
Bcl2a1	B-cell leukemia/lymphoma 2 related protein A1	Bcl-2family	11.75	<0.0001
Bcl2l11	BCL2-like 11(apoptosis facilitator)	Bcl-2family	3.07	0.0053
Birc1b	Baculoviral IAP repeat-containing 1b	IAP family	5.07	<0.0001
Birc3	Inhibitor of apoptosis protein 1	IAP family	47.5	<0.0001
birc5	Baculoviral IAP repeat-containing 5	IAP family	-2.3	0.004
Card6-predicted	Caspase recruitment domain, member 6	Caspase family/Card family	-2.2	0.0114
Casp14-predicted	Caspase 14 (predicted)	Caspase family	22.35	<0.0001
Casp9	Caspase 9	Caspase family/p53 family/ Card family	-2.13	0.0231
Cflar	CASP8 and FADD-like apoptosisregulator	Death domain/Death effector domain family	2.81	0.0002
Cradd	CASP2 and RIPK1domain containing adaptor with death domain(predicted)	Death domain family/Card family/p53 family	-2.11	0.0009
Gadd45a	Growth arrest and DNA-damage-inducible 45 alpha	p53familu	6.82	<0.0001
Il10	Interleukin10	NF-kappa Bfamily	21.65	<0.0001
Lhx4-predicted	LIM homeobox protein 4(predicted)	LIM-homeobox gene family	11.7	<0.0001
Lta	Lymphotoxin A	TNFligand family/NF-kappa B family	26.83	<0.0001
Nfkb1	Nuclear factor of kappa light chair gene enhancer in B-cells1, p105	p53family/NF-kappa B family	7.15	<0.0001
Prok2	Prokineticin 2 precursor	TNF ligand/receptor family	19.89	<0.0001
Pycard	Apoptosis-associated speck-like protein containing a CARD	Death domain family/ p53family/caspase family	2.42	<0.0001
Ripk2	Receptor(TNFRSF)-interacting serine-threonine kinase 2, anti-apoptotic	Card family	4.15	<0.0001
Tnf	Tumor necrosis factor superfamily, member 2	TNF ligand family/ NF-kappa B family	25.71	<0.0001

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Apoptosis is an occurring process to direct programmed cell self-destruction. Apoptosis generally can occur through two major broad pathways: extrinsic and intrinsic pathways. The extrinsic pathway begins outside of the cell when the cell determines to die as lethal factors appear in extracellular environment, whereas the intrinsic pathway begins when an injury occurs within the cell. In our previous studies, we demonstrated that mefloquine-induced apoptotic signaling in the cochlear hair cells and spiral ganglion neurons were associated with activation of numerous caspases, particularly the typical involvement of the mitochondrial pathway with caspase-9 activation and cell death receptor-

Gene	Description	Functional Gene Grouping	Fold changes	pvalue
Tnfrsf10b–predicted	Tumor necrosis factor receptor superfamily, member 10b(predicted)	TNF receptor family/p53 family/NF–kappa B	2.54	0.0017
Tnfrsf11b	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	TNF receptor family/ Death domain family	5.5	<0.0001
Tumor necrosis factor receptor superfamily, member 1a	Tumor necrosis factor receptor superfamily, member1a	TNF receptor family	2.55	0.0037
Tnfrsf6	Tumor necrosis factor receptor superfamily, member6	TNF receptor family	2.84	0.0006
Tnfsf10	Tumor necrosis factor(ligand) superfamily, member 10	TNF ligand family	–3.83	<0.0001
Tnfsf12	Tumor nectosis factor(ligand) superfamily, member12	TNF ligand family	–2.09	0.0049
Tnfsf5	Tumor necrosis factor(ligand) superfamily, member5	TNF ligand family	15.35	<0.0001
Tnfsf6	Tumor necrosis factor(ligand) superfamily, member6	TNF ligand family	5.23.	<0.0001
Tradd	TNFRSF1A–associated via death domain	p53 family/NF–kappa B family /Death domain	–2.06	0.0002
Trp63	Transformation related protein 63	p53 family	3.23	0.0005
Trp73–predicted	Transformation related protein73(predicted)	p53 family	6.68	0.0001

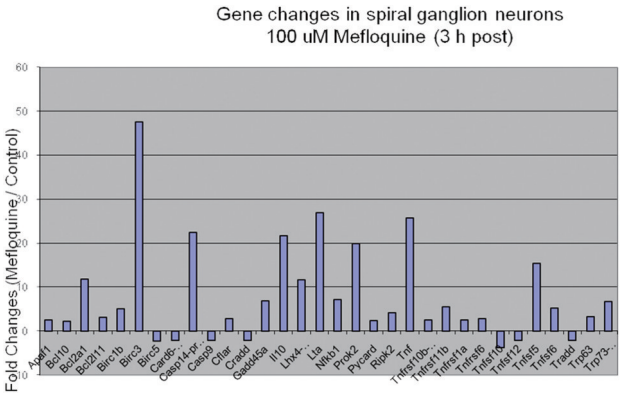

Figure 2 Significant changes in gene expression in cochlear spiral ganglion neurons 3 hours after 100 μM mefloquine treatment. The expression in 25 genes was increased, but the expression in another 7 genes was decreased

mediated signaling pathway with caspase–8 activation.[12, 13, 19–21] This indicates that the intracellular organs, such as mitochondria and cell death receptors, were injured internally by mefloquine. Generally speaking, mefloquine–induced intracellular apoptotic signaling following caspase activation should be classified as both extrinsic, involving responses from cell death receptors on the cell membrane, and intrinsic, involving responses from mitochondrial apoptotic pathway.

The findings in the current study show that numerous genes and multiple apoptotic pathways are involved in mefloquine–induced apoptosis in both cochlear epithelium and spiral ganglion neurons. The early responding apoptotic genes in response to mefloquine injury mostly involve the Bcl–2 family, Caspase family, Card family, TNF ligand and TNF receptor family, IAP family, Death domain and Death effector domain family, DNA damage and p53 family, and NF–kappa B family. According to their functional configurations, one gene directly or indirectly belongs to or is related to several apoptotic families. Therefore, altered genes may arouse multiple apoptotic pathways. To better understand potential relations among these functional gene groupings, we examined interactions among the responding genes. Most responding genes in the TNF ligand / TNF receptor family are

also crucial members in the DNA damage / p53 family, Death domain / Death effector domain family, and NF-kappa B family. [22] All members in the Card family possess the caspase recruitment domain which is undoubtedly linked to the Caspase family. In addition, all members in the IAP family serve as endogenous inhibitors of programmed cell death, especially binding and inhibiting activation of caspases to prevent apoptosis. Therefore, the IAP family is characterized as worthy opponent of caspases, and its activation indirectly reflects caspases in a state of action. Apoptosis regulator Bcl-2 family governs mitochondrial outer membrane permeability. Thus, changes in gene expression in the Bcl-2 family are thought to be in connection with mitochondrial degeneration. Moreover, many genes in the Bcl-2 family are also crucial members as strong prognostic indicators related to p53 signaling pathway. [23] The p53 pathway and its complex are hereby believed to interact with the Bcl-2 family as well. [24, 25] The mitochondrial pathway of apoptotic functions can be also triggered in response to various intracellular stress, such as the p53 signaling pathway, DNA damage, Death receptor stimulations, as well as reversed stimuli from the TNF ligand / receptor family, which triggers activation of a caspase cascade that shifts the balance in the Bcl-2 family. [26, 27] All factors and approaches should be taken into considerations in apoptotic signaling in the mitochondrial pathway, including its upstream, downstream, and other surrounding pathways.

In our previous studies, we have shown that mefloquine damages rat cochlear hair cells and spiral ganglion neurons in a dose-dependent manner. In addition, the initiator caspase-8 was activated 24 h after 35 μM mefloquine treatment in response to apoptotic signaling from the cell death receptors in the cell membrane. Another initiator caspase-9 was also activated in response to cytochrome c release from damaged mitochondria, which interacted with Apaf-1, causing self-cleavage of caspase-9. These two initiator caspases, caspase-8 and caspae-9 then activated their downstream executioner caspase-3 to cleave various targets leading to cell apoptosis. These findings suggest that mefloquine-induced apoptotic signals in caspase pathway may initiate from two aspects: damage to the cell membrane and damage to the mitocho-ndria. [12, 19, 21] Using RT-PCR apoptosis-focused gene array to detect changes in apoptosis-related genes 3 hours post-100 μM mefloquine, we found that nine of the 23 responding genes in the cochlear basilar membrane, and twelve of the 32 responding genes in the spiral ganglion neurons belonged to or were related to the tumor necrosis factor supper family, consistent with our previous findings in caspase-8 activation 24 hours after mefloquine treatment. [2, 19, 21] In addition, numerous responding genes directly belong to or are indirectly related to the apoptotic mitochondrial pathway, such as *Apaf1*, *Bcl2a1, BCl2l11, Bid3, Birc1b, Birc3, Birc4, Bnip1, Casp1, Casp7, Casp9, Cradd, Mcl1, TNF,* etc. These responding genes are in conformity with the post-mefloquine caspase-9 activation reported in our previous publications. [12, 19, 21] Therefore, the tumor necrosis factor pathway and the mitochondrial pathway are considered to be the major apoptotic pathways in mefloquine-induced cell apoptosis in the cochlear basilar membrane and spiral ganglion neurons. Although changes in gene expression showed a significant overlap between the cochlear basilar membrane and spiral ganglion neurons, indicating that the major apoptotic pathways in the two structures, differences exist which may reflect the unique response in each tissue.

Changes in a wide range of apoptotic and anti-apoptotic signals in the cochlear basilar membrane (hair cells and supporting cells) and spiral ganglion neurons are observed in the current study. Changes in apoptosis-related gene expression suggest potential changes at mRNA level in response to mefloquine-induced early apoptotic injury. While infringement of mefloquine can alter gene expression, it may not affect the function of the proteins made by the corresponding genes at the same time. Recently, we have assayed changes in the same model treated with 100 μM mefloquine for 3 hours, using an apoptosis-related antibody microarray. The proteomic analysis revealed much less expressive changes in apoptosis-related signaling proteins than in mRNA, [28] indicating that gene changes always take precedence of functional changes in protein. It may also suggest a possibility of important translational control of the balance between the anti-apoptotic and the apoptotic responses. In our proteomic studies, some responding apoptosis-related proteins were a response or partial response to caspases activities and changes of gene ex-

pressions. For example, one of the responding proteins, mitogen-activated protein kinase, was greatly increased, suggesting the kinase cascade due to apoptotic signal stimulationfrom the cellular surface to the DNA in the nucleus. [29] This result is definitely consistent with caspase-8 activity and nuclear destruction we discovered in our previous studies [12, 19, 21] and with changes in gene expression in the TNF ligand / receptor family, caspase family, and DNA damage / p53 super family observed in the current study. In addition, decreased Caspase-3 protein expression at 3 hours post-mefloquine [28] may represent temporary suppression of executioner caspase-3 due to increased activities of anti-apoptotic genes in the IAP family and Bcl-2 family at the early stage of apoptosis.

In summary, the data from this study indicate that numerous pro-apoptotic and anti-apoptotic genes in the cochlear epithelium and spiral ganglion neurons are rapidly activated in response to mefloquine-induced apoptosis. Although multiple apoptotic pathways may be aroused by mefloquine injury, the death receptor-mediated signaling pathway and the mitochondrial pathway seem to be the major accesses of apoptotic pathways in mefloquine-induced cochlear apoptosis.

Refrences

1 Sturchler D, Handschin J, Kaiser D, et al. "Neuropsychiatric side effects of mefloquine." N Engl J Med, 1990, 322 (24): 1752-1753.

2 Weinke T, Trautmann M, Held T, et al. "Neuropsychiatric side effects after the use of mefloquine." Am J Trop Med Hyg, 1991, 45(1): 86-91.

3 Hennequin C, Bouree P, Bazin N, et al. "Severe psychiatric side effects observed during prophylaxis and treatment with mefloquine." Arch Intern Med, 1994, 154(20): 2360-2362.

4 Phillips-Howard PA. and ter Kuile FO. "CNS adverse events associated with antimalarial agents. Fact or fiction?" Drug Saf, 1995, 12(6): 370-383.

5 Croft AM. and World MJ. "Neuropsychiatric reactions with mefloquine chemoprophylaxis." Lancet, 1996, 347(8997): 326.

6 Schlagenhauf P. "Mefloquine for malaria chemoprophylaxis 1992-1998: a review." J Travel Med, 1999, 6(2): 122-133.

7 Rendi-Wagner P, Noedl H, Wernsdorfer G, et al. "Unexpected frequency, duration and spectrum of adverse events after therapeutic dose of mefloquine in healthy adults." Acta Trop, 2002, 81(2): 167-173.

8 McArdle JJ, Sellin LC, Coakley KM, et al. "Mefloquine selectively increases asynchronous acetylcholine release from motor nerve terminals." Neuropharmacology, 2006, 50(3): 345-353.

9 Dow G, Bauman R, Caridha D, et al. "Mefloquine induces dose-related neurological effects in a rat model." Antimicrobial Agents and Chemotherapy, 2006, 50(3): 1045-1053.

10 McArdle JJ, Sellin LC, Coakley KM, et al. "Mefloquine inhibits cholinesterases at the mouse neuromuscular junction. " Neuropharmacology, 2005, 49(8): 1132-1139.

11 Fusetti M, Eibenstein A, Corridore V, et al. "Mefloquine and ototoxicity: a report of 3 cases." Clin Ter, 1999, 150(5): 379-382.

12 Ding D, Qi W, Yu D, et al. "Ototoxic effects of mefloquine in cochlear organotypic cultures." Journal of Otology, 2009, 4(2): 29-38.

13 Yu D, Ding D, Jiang H, et al. "Mefloquine Damage Vestibular Hair Cells in Organotypic Cultures." Neurotox Res, 2010..

14 Ding D, Wang P, Jiang H, et al. "Gene expression in cisplatin ototoxicity and protection with p53 inhibitor." Chinese Journal of Otology, 2009, 4(2): 15-24.

15 Ding D, Jiang T, Qi W, et al. "Science of the inner ear." Chinese Science and Technology Publishing Company. 2010, Page 39-49.

16 Jiang H, Ding D, Salvi R. "Mefloquine-induced changes in apoptotic gene expression in cochlear basilar membrane and spiral ganglion neurons " Abstr Assoc Res Otolaryngol, 2008.

17 Hu BH, Cai Q, Manohar S, et al. "Differential expression of apoptosis-related genes in the cochlea of noise-exposed rats." Neuroscience, 2009, 161(3): 915-925.

18 Wei L, Ding D, Salvi R. "Salicylate-induced degeneration of cochlea spiral ganglion neurons-apoptosis signaling." Neuroscience, 2010, 168(1): 288-299.

19 Ding D, Qi W, Jiang H, et al. Mefloquine induced apoptosis in hair cells and spiral ganglion neurons in cochlear organotypic cultures. Abstr Assoc Res Otolaryngol, 2007.

20 Yu D, Ding D, Salvi R. Ototoxicity of mefloquine in vestibular organotypic cultures. Abstr Assoc Res Otolaryngol, 2008.

21 Ding D, Jiang T, Qi W, et al. "Science of the inner ear." Chinese Science and Technology Publishing Company, 2010, 302-307.

22 Wang Y, Sun X, Wu J, et al. "Casein kinase 1alpha interacts with RIP1 and regulates NF-kappaB activation." Biochemistry, 2008, 47(1): 441-448.

23 Silvestrini R, Veneroni S, Daidone MG, et al. "The Bcl-2 protein: a prognostic indicator strongly related to p53 pro-

tein in lymph node-negative breast cancer patients." J Natl Cancer Inst, 1994, 86(7): 499-504.

24 Park SA, Park HJ, Lee HJ, et al. "Bcl-2 blocks cisplatin-induced apoptosis by suppression of ERK-mediated p53 accumulation in B104 cells." Brain Res Mol Brain Res, 2001, 93 (1): 18-26.

25 Palacios G, and Moll UM. "Mitochondrially targeted wild-type p53 suppresses growth of mutant p53 lymphomas in vivo." Oncogene, 2006, 25(45): 6133-6139.

26 Haupt S, Berger M, Goldberg Z, et al. "Apoptosis - the p53 network." J Cell Sci , 2003, 116(Pt 20): 4077-4085.

27 Haupt S, Louria-Hayon I, Haupt Y. "P53 licensed to kill? Operating the assassin." J Cell Biochem, 2003, 88(1): 76-82.

28 Ding D, Jiang H, He J, et al. "Proteomic Analysis of Mefloquine Ototoxicity." Abstr Assoc Res Otolaryngol, 2009.

29 Dhillon AS, Hagan S, Rath O, et al. "MAP kinase signalling pathways in cancer." Oncogene, 2007, 26(22): 3279-3290.

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